IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

ULF TILSTAM ET AL.

Examiner: Howard V. Owens, Jr.

Serial No.: 09/471,040

Group Art Unit: 1623

Filed: December 23, 1999

Title: PROCESS FOR THE PRODUCTION OF FLUDARABINE-PHOSPHATE LITHIUM, SODIUM, POTASSIUM, CALCIUM AND MAGNESIUM SALTS AND PURIFICATION PROCESS FOR THE PRODUCTION OF FLUDARABINE-PHOSPHATE AND FLUDARABINE-PHOSPHATE WITH A PURITY OF AT LEAST 99.5%

DECLARATION UNDER 37 CFR §1.132

We Thomas Wessa and Harald Rabe, both being duly warned, declare that:

Our curricula vitae are attached demonstrating our expertise to make this declaration.

We are familiar with the above-identified application, the office action of July 5, 2005, Montgomery et al. USP 4,357,324 and Blumberg et al. USP 5,109,919.

We are employees of the assignee of this application, Schering AG, Berlin, Germany.

In efforts to reproduce example II of USP 4,357,324 to Montgomery et al., the following experiments were performed by us or under our supervision.

Preliminary Matters

Example II of Montgomery mentions extraction of a product with a mixture of SOEtOH:NH_2OH:$9H_2O$. This nomenclature is ambiguous. It is our understanding that "SO" means "solvent." "EtOH" means ethanol, as is conventional. "NH_2OH," we assume, most conventionally would mean NH_4OH, i.e., ammonia water. Nevertheless, another, less likely, possibility is that the expression is meant to refer to hydroxylamine (NH_2OH). Consequently, we have performed the Montgomery process using each of the two possibilities as extractant, i.e., Experiment I employed ammonia water and Experiment II employed hydroxylamine.

Example II of Montgomery also refers to a "Domex" column. This is an obvious typographical error. Clearly, the conventional column, "DOWEX" was meant.

In addition, certain amounts and conditions of reagents are not specified in Montgomery. In all such cases, common sense, scientifically conventional conditions were employed, as indicated.

Experiment 1

(Montgomery process, using ammonia for extraction)

Montgomery Example II was reproduced in 3.85 scale (to enhance product amount).

10g of 9-β-D-arabinofuranosyl-2-fluoroadenine was added to 88.5 ml triethylphosphate. The mixture was cooled to 0°C and 17.7 g $POCl_3$ was added. Afterwards, the mixture was stirred for 3.5 hours. Then the mixture was poured into 770 ml of ice water. The pH of the solution was adjusted to pH=2 using 54 ml of 6N NaOH. Subsequently, the mixture was extracted by chloroform (2 x 692 ml). The aqueous layer was slurried with 154 g charcoal and 77 g celite for 20 minutes. After filtration of the mixture, the solids were washed with water (4 l) until free of acid. Fludarabine phosphate, crude was extracted from the neutralized solids using 2 l of a mixture of $EtOH:NH_4OH:9H_2O$. The eluate was split into two parts. One part was lyophilized, yielding 3.3 g (purity 76.9%). The other part was kept for reproduction.

50 g Dowex 1 x 8 ion exchange resin was slurried in water and a column was prepared. The column was rinsed with 200 ml water. The ion exchange column was transferred into the formate form using 2 l formic acid (1mol/l). Afterwards the column was washed with 1.5 l water. 0.5 g fludarabine phosphate as described above dissolved in 5 ml water was placed onto the column. The product was extracted using different concentrations of hydrochloric acid obtaining 17 fractions (see table I). The concentrations and volumes were chosen conventionally in order to get the product off the column. (No procedure was given in the patent.)

Fraction 8 is the fraction for the fludarabine phosphate peak. (Fludarabine phosphate is also termed "Fludara" herein and in the tables.) It can be seen that this has a purity of 82.67% (100% HPLC method).

Experiment 2

(Montgomery process, using hydroxyl amine for extraction)

Montgomery Example II was reproduced in 1.93 scale (to enhance product amount). 5g of 9-β-D-arabinofuranosyl-2-fluoroadenine was added to 44.3 ml triethylphosphate. The mixture was cooled to 0°C and 8.9 g $POCl_3$ was added. Afterwards, the mixture was stirred for 3.5 hours. Then the mixture was poured into 385 ml of ice water. The pH of the solution was adjusted to pH=2 using 25 ml of 6N NaOH. Subsequently, the mixture was extracted by

chloroform (2 x 346 ml). The aqueous layer was slurried with 77 g charcoal and 38.5 g celite for 20 minutes. After filtration of the mixture, the solids were washed with water (14 l) until free of acid. Fludarabine phosphate, crude was extracted from the neutralized solids using 2 l of a mixture of EtOH:NH_2OH:$9H_2O$. The extract was lyophilized, yielding 7.4 g (purity 70.4 %).

50 g Dowex 1 x 8 ion exchange resin was slurried in water and a column was prepared. The column was rinsed with 200 ml water. The ion exchange column was transferred into the formate form using 2 l formic acid (1mol/l). Afterwards the column was washed with 1.5 l water. 0.5 g fludarabine phosphate as described above dissolved in 10 ml water was placed onto the column. The product was extracted using different concentrations of hydrochloric acid, obtaining 17 fractions (see Table II). The concentrations and volumes were chosen conventionally in order to get the product off the column. (No procedure was given in the patent.)

Fraction 8 is the fraction for the fludarabine phosphate peak. It can be seen that this has a purity of 87.24% (100% HPLC method).

Experiment 3

(Additional experiment using ammonia for extraction)

In addition to these experiments, we also performed a third experiment which varied the conditions of Montgomery somewhat in accordance with conventional lab practices. This was done to ensure that the Montgomery process was fairly reproduced. Whereas the purity increased to 94.90%, like the purities achieved in Experiments I and II, none met the requirement of this invention of a purity of at least 99.5%.

Montgomery Example II was reproduced on a x 3.85 scale (to obtain a larger raw batch for purification testing) with variations described below.

10g of 9-β-D-arabinofuranosyl-2-fluoroadenine and 88.5 ml triethylphosphate were suspended under stirring at a temperature of 0°C. To this suspension 17.5g of $POCl_3$ was added dropwise at a temperature of 1.5°C to 2.5°C. The reaction mixture was stirred for 3.5 h beginning at 0°C to 5°C.

The clear solution was placed into 770ml of ice cold water and the pH of the solution was adjusted to pH=2 by the addition of 48ml of 6 N sodium hydroxide solution.

The reaction product was extracted twice with 500ml chloroform (2 x 250ml). To the slightly turbid aqueous phase 155 g of charcoal and 77g of Celite were added and the resulting

mixture was stirred for 20 minutes. The resulting solid compound was separated and washed to be free of acid, up to pH=4, with 16 l of purified water.

The Celite/charcoal mixture was placed on a fritted glass filter and eluted with a mixture of 200 ml ethanol, 200 ml of ammonia (25%) and 1800 ml of purified water. The eluate was lyophilized. The yield was 7.6 g (59.3% of theory) of fludarabine phosphate, having a purity of 78.02% (100% HPLC method).

1g of the so produced fludarabine phosphate was dissolved in 30 ml purified water, using a 250 ml flask. 2g of the ion exchanger Dowex 1x8 was added to the solution and the reaction mixture was stirred at room temperature. After this, the loaded ion exchanger was separated.

In order to liberate fludarabine phosphate from the ion exchanger, the ion exchanger was treated with 60ml of 0.1 N hydrochloric acid. The mixture was stirred for 1h.

The ion exchanger was separated again and part of the filtrate (approx. 50%) was concentrated to dryness. Fludarabine phosphate (183mg) was obtained as a colorless solid having a purity of 88.73% (according to 100% HPLC method).

Separately, 50 g Dowex 1 x 8 ion exchange resin was slurried in water and a column was prepared. The column was rinsed with 200 ml water. The ion exchange column was transferred into the formate form using 2 l formic acid (1mol/l). Afterwards the column was washed with 1.5 l water. 0.5 g fludarabine phosphate as described above dissolved in 10 ml water was placed onto the column. The product was extracted using different concentrations of hydrochloric acid, obtaining 14 fractions (see Table III). The concentrations and volumes were chosen conventionally in order to get the product off the column. (No procedure was given in the patent.)

Fraction 7 is the fraction for the fludarabine phosphate peak. It can be seen that this has a purity of 94.90% (100% HPLC method).

Experiment 4

(Additional experiment concerning the OD29 document (Declaration of Dr. Knill) from corresponding European case EP1047704)

The examiner postulates that any of the fludarabine phosphate products having the stated purities could be improved in purity by simply recycling the product derived from a column into another stage of the same or a different column. This point is indeed correct, insofar as it states that a highly pure fludarabine phosphate could be obtained in a liquid

solution resulting from such a multistage (or even, perhaps, appropriate single stage) column. Indeed, in a scientific report submitted during the Opposition Proceeding concerning the corresponding European patent (1047704), such a high purity was discussed to be achieved on a certain Altech Alltima C18 column (> 99.95% purity). (Corcoran Declaration)
In a further test report, submitted during the Opposition Proceedings, concerning the corresponding European patent (1047704), which we are informed is official document OD29, it was mentioned that a pure fludarabine phosphate can be produced according to well known methods. The method of this Declaration of Dr. Knill (OD29) comprises the purification of fludarabine phosphate on a reversed phase C-18 silica column at atmospheric pressure to remove all impurities, and collecting the fludarabine phosphate off the column using a 50:50 acetonitrile:water mixture, washing and removing the acetonitrile with a stream of nitrogen gas, and removing the water for injection at room temperature under vacuum, leaving the purified fludarabine phosphate as a crystalline solid. Then the fludarabine phosphate was reconstituted in water for injection and injected as a sample onto a Alltech Alltima C18, 250x4.6 mm, 5 μm column. We have thoroughly analysed the described process and reproduced the important portion of it.The result always was the production of fludarabine phosphate which has a purity of less than 99.5%.

As noted above, it is possible to prepare liquid samples of fludarabine phosphate having a purity of ≥ 99.5%. Thus, our experiments started with such solutions. From there we reproduced the methods of OD29 as closely as possible. However, for some reason, many details are omitted from Dr. Knill's declaration, in particular the concentration of the purified fludarabine phosphate in the acetonitride/water for injection (ACN/WFI) 50/50 v/v solution. Thus, we employed a range of reasonable possible concentrations to ensure the experimental parameters of OD29 were encompassed.

According to the method described in OD29, we have estimated the possible concentrations of fludarabine phosphate collected off the column. The concentrations were calculated on the basis of standard flow rates (typically: 1ml/min), typical elution volumes (pure fraction= 1ml, raw fraction = 5ml and in between = 3ml), and the stated injection volume (20μl) of the stated 5mg/ml sample solution. As a result, we employed amounts of 20 μg in 1, 3 or 5 ml of solution as the possible reasonable range of collection samples. See the second and third columns of Table IV.

All dilutions have been produced from a concentrated standard solution which has been accordingly diluted with an acetonitrile/ water (1:1) mixture as discussed in OD29. For

the production of the standard solution, 16 mg of highly pure fludarabine phosphate were placed in 100ml of an acetonitrile/water for injection (1:1) mixture and was dissolved by using an ultrasound water bath for 2 minutes. As shown in the first row of Table IV, this solution had a purity of 99.67%.

Per OD29, nitrogen gas was blown into the fludarabine phosphate solution to remove the acetonitrile, and the water was removed under vaccum . Additionally, other samples were dried in a drying cabinet at room temperature over night.

No crystals of fludarabine phosphate could be seen visually in the dried samples due to the small amounts involved. However, it is assumed that crystals were formed. Per OD29, these crystals were reconstituted in water for injection and their purity was determined, all according to standard methods, i.e., using the procedures of the European Pharmacopeoia. (See OD30 of the European Opposition.) The results are shown in Table IV. As can be seen, in all cases the fludarabine phosphate had a purity well less than 99.5%. Thus, it is not possible to obtain a purity $\geq$ 99.5% for crystalline/fludarabine phosphate using the method of Dr. Krill in OD29.

Experiment 5

(Behavior of fludarabine phosphate when using different amounts of substance)

In a further experiment, the behaviour of the same highly pure standard solution of fludarabine phosphate was further analysed, using different absolute amounts of fludarabine phosphate. For this, different amounts of fludarabine phosphate were dissolved in water:acetonitrile 1:1. However, the concentration of the solutions was always the same. See Table V. The result is that, independently of the used amounts of fludarabine phosphate, the sum of the decomposition products increased in comparison to the originally applied pure fludarabine phosphate. The results are shown in Table V. This experiment shows that amount variations in Experiment 4 above would not affect the results.

Experiment 6

(Instability of fludarabine phosphate under various physical conditions; conventional purification methods are thus inappropriate)

As shown in experiments 4 and 5, impurities in fludarabine phosphate cannot be separated by simply applying the compound onto an HPLC column and recycling the solution

with the compound until it is pure, since fludarabine phosphate is instable in solution. Thus, fludarabine phosphate cannot be simply isolated from the eluent.

The stability problem is further established in the attached Test Report (Experiment 6; pages 15-20 hereof).

We hereby confirm that all statements in the attached Test Report are true and accurate.

The Test Report demonstrates that fludarabine phosphate is an especially unstable compound in solid form. It decomposes at a temperature of 50°C and 60°C with normal humidity. With increased humidity, more significant decomposition occurs. This is demonstrated in the table on pages 15-18 of the enclosed Test Report. Thus, even under normal humidity conditions at 25°C, 50°C, 60°C, etc., the sum of contaminants is still 0.91, 1.10 and 1.12%, respectively. As soon as humidity is raised, e.g., to 75%, the sum of contaminants increases at 50° to 1.40% and at 60° to 11.78%. Values for 70° and 90° are also included and show even more significant contamination. Clearly, exposure to water significantly enhances the instability of fludarabine phosphate. The data in section 3 of the enclosed Test Report (pages 18-20) demonstrate a similar high instability of fludarabine phosphate at acidic pHs and at alkaline pHs. Similarly, significant decomposition can be seen at neutral pH 7.

As stated in the summary on page 21 of the enclosed Test Report, very strong decomposition of fludarabine phosphate takes place in aqueous solution under acidic or alkaline conditions. Even in the neutral pH range, there is clear decomposition. Similarly, at elevated temperatures and high humidity, significant decomposition occurs.

This unusual instability is well known to those of skill in the field. For example, Ash Stevens, Inc., Detroit, Michigan is a commercial supplier of fludarabine phosphate to Schering AG, the NDA holder for this active ingredient. In its US patent, 5,110,919 to Blumbergs et al., Ash Stevens states:

> In view of the extensive handling in the last step, a final recrystallization was necessary to remove any inadvertently-introduced water-insoluble impurities. The acidic product is, however, unstable in hot water. Some decomposition occurs during the recrystallization and no real improvement in purity results. With careful handling in the last step, it is possible that the final recrystallization can be avoided. (Column 10, line 67-Column 11, line 6.)

What all of this means for the examiner's question regarding recycling or repetition of ion exchange purification, is that all conventional techniques used to prepare "pure" crystalline fludarabine phosphate inevitably used conditions of temperature/humidity/aqueous

solution/solvent solution/pH, which caused fludarabine phosphate to decompose, thereby producing only products having purities less than 99.5%. This has been proven by the data in the Test Report demonstrating the strong decomposition which occurs at all reasonable conditions which would be used in conventionally purifying fludarabine phosphate.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Thomas Wessa

(Dr. Thomas Wessa)

Dated: 23.02.06

H. Rabe

(Dr. Harald Rabe)

Dated: 23.02.06

Table I - Experiment 1 (Montgomery process, using ammonia for extraction)

Fraction No.	Volume of the Fraction [ml]	HCl - Gradient (concentration of HCl) [Mol / l]	Fludara Purity [%]	∑ Impurities [%]	6-Amino-9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-2-ol [%]	6-Amino-9H-purin-2-ol [%]	9-(3,5-O-diphosphono-ß-D-arabinofuranosyl)-2-fluoro-9H-purin-6-amine [%]	2-Fluoro-9H-purin-6-amine [%]	2-Fluoro-9-(ß-D-arabinofuranosyl)-9H-purin-6-amine [%]	2-Ethoxy-9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-6-amine [%]
F1	100	0								
F2	50	0,05			0	0	0	0	0	0
F3	50	0,1			0	0	0	0	0	0
F4	50	0,2			0	0	0	0	0	0
F5	50	0,3								
F6	50	0,5	2,5		6,6	0,18				5,1
F7	50	0,75	74,9	25,1	9,92	1,95	11,23	0,07		2,11
F8	50	0,75	82,67	17,33	4,76	0,35	19,51	0,07		0,43
F9	100	1	81,27	18,73	5,24	0,2	24,26	0,09		0,32
F10	100	1	80,5	19,5	5,72	0,08	26,24	0,12		0,34
F11	100	1	78,16	21,84	6,4		28,18	0,28		0,58
F12	100	1	74,45	25,55	4,84		34,16	0,26		
F13	100	1	73,44	26,56	4,56		36,94	0,25		
F14	100	1	72,47	27,53	4,08		38,78	0,25		
F15	100	1	71,38	28,62	3,72		42,07			
F16	100	1	70,42	29,58	1,92		45,22			
F17	100	1	69,62	30,38			51,19			

The sum of impurities is lower in contrats to the single impurities with respect to response factor calculation according to Pharmacopoe

Table II - Experiment 2 (Montgomery process using hydroxyl amine for extraction)

Fraction No.	Volume of the Fraction [ml]	HCl - Gradient (concentration of HCl) [Mol / l]	Fludara Purity [%]	Fludara Impurities [%]	6-Amino-9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-2-ol [%]	6-Amino-9H-purin-2-ol [%]	9-(3,5-O-diphosphono-ß-D-arabinofuranosyl)-2-fluoro-9H-purin-6-amine [%]	2-Fluoro-9H-purin-6-amine [%]	2-Fluoro-9-(ß-D-arabinofuranosyl)-9H-purin-6-amine [%]	2-Ethoxy-9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-6-amine [%]
F1	100	0,00	26,54	73,46	0	86,6				
F2	50	0,14	0	100		164,15				
F3	50	0,28	37,28	62,72		67,15				
F4	50	0,56	0	100	333,96					
F5	50	0,83	0	100	35,84		0,53	2,74		
F6	50	1,39	42,56	57,44	46,16		7,66	0,39	0,17	
F7	50	2,09	84,81	15,19	8,92	0,08	7,32	0,09	0,06	
F8	50	2,09	87,24	12,76	6,16		11,04	0,1	0,05	
F9	100	2,78	86,58	13,42	6,92		13,91	0,13	0,04	
F10	100	2,78	83,16	16,84	5,32		16,19	0,2		
F11	100	2,78	81,55	18,45	4,76		19,76	0,21		
F12	100	2,78	77,62	22,38	3,52		25,37	0,22		
F13	100	2,78	72,32	27,68			32,26			
F14	100	2,78	73,75	26,25			35,89			
F15	100	2,78	63,16	36,84			47,79			
F16	100	2,78	70,52	29,48			39,98			
F17	100	2,78	52,27	47,73			55,92			

The sum of Impurities is lower in contrast to the single impurities with respect to response factor calculation, according to pharmacopoe

Table III - Experiment 3 (Additional experiment using ammonia for extraction)

Fraction No. (Different fractions of the gradient Elution Pattern)	Volume of the fraction [ml]	Not determinated (%)	Not determinated (%)	Not determinated (%)	Not determinated (%)	Fludara Purity [%]	Fludara content in Fraction [mg]
#12FL32F**1** – H2O	100	0,00	0,00	0,00	0,00	0,00	0,0
#12FL32F**2** – 0.05 HCl	50	0,00	0,00	0,00	0,00	0,00	0,0
#12FL32F**3** - 0.10 HCl	50	0,00	0,00	0,00	0,00	0,00	0,0
#12FL32F**4** – 0.20 HCl	50	0,00	0,00	0,00	0,00	0,00	0,0
#12FL32F**5** – 0.30 HCl	50	0,00	0,00	100,00	0,00	0,00	0,0
#12FL32F**6** – 0.50 HCl	50	0,00	0,00	99,11	0,00	0,89	0,1
#12FL32F**7** – 1.00 HCl	100	0,12	0,24	0,60	4,14	94,90	149,2
#12FL32F**8** – 1.00 HCl	100	0,59	0,00	0,09	12,66	86,65	81,4
#12FL32F**9** – 1.00 HCl	100	0,00	0,00	0,00	17,61	82,39	25,2
#12FL32F**10** – 1.0 HCl	100	0,00	0,00	0,00	20,52	79,48	11,7
#12FL32F**11** - 1.0 HCl	100	0,00	0,00	0,00	19,01	80,99	6,9
#12FL32F**12** - 1.0 HCl	100	0,00	0,00	0,00	25,23	74,77	4,1
#12FL32F**13** - 1.0 HCl	100	0,00	0,00	0,00	34,39	65,61	1,8
#12FL32F**14** - 1.0 HCl	100	0,00	0,00	0,00	26,92	73,08	1,2
							281,4
						Brought on the column:	**286,7**

Table IV – Experiment 4(Additional experiment concerning the OD29 reference in European case 1047704)

Example-No.	Fludarabine phosphate concentration	Eluting solvent (Acetonitrile/ Water) 1:1	6-Amino- 9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-2-ol [%]	6-Amino-9H-purin-2ol [%]	9-(3,5-O-diphosphono-ß-D-arabinofuranosyl)-2-fluoro-9H-purin-6-amine [%]	2-Fluoro-9H-purin-6-amine [%]	2-Fluoro-9-(ß-D-arabinofuranosyl)-9H-purin-6-amine [%]	2-Ethoxy-9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-6-amine [%]	additional not determined impurities [%]	All impurities [%] *)
(Standard)			**0,23**	**0,05**	**<0,01**	**0,01**	**<0,01**	**<0,01**	**0,04**	**0,33**
1	20 µg	1 ml	0,69	0,20	0,07	0,20	0,04	<0,01	0,58	1,78
2	20 µg	3 ml	0,56	0,04	0,05	0,18	0,06	<0,01	1,41	2,30
3	20 µg	5 ml	1,44	<0,02	0,07	0,19	0,07	<0,01	1,63	3,40
4	80 µg	1 ml	3,40	2,27	<0,01	0,27	0,04	<0,01	1,04	7,02
5	80 µg	3 ml	0,60	0,12	0,04	0,11	0,07	<0,01	0,61	1,55
6	80 µg	5 ml	0,62	0,10	<0,01	0,13	0,09	<0,01	0,54	1,48
7	40 µg	1 ml	2,27	0,44	<0,01	0,15	0,04	<0,01	0,80	3,70
8	40 µg	3 ml	0,71	0,06	0,02	0,14	0,06	<0,01	0,74	1,73
9	40 µg	5 ml	0,47	0,14	0,09	0,22	0,13	<0,01	1,09	2,14
10	160 µg	1 ml	5,14	3,43	<0,01	0,25	0,03	<0,01	1,43	10,28

*) Values of <0,01 and <0,02 not considered

Table V – Experiment 5 (Behaviour of fludarabine phosphate when using different amounts of substance)

Example-No.	Fludarabine phosphate concentration [mg/ml water: acetonitrile 1:1]*)	6-Amino- 9-(5-O-phosphono-ß-D-arabinofuranosyl)- 9H-purin-2-ol [%]	6-Amino-9H-purin-2ol [%]	9-(3,5-O-diphos-phono-ß-D-ara-binofuranosyl)-2-fluoro-9H-purin-6-amine [%]	2-Fluoro-9H-purin-6-amine [%]	2-Fluoro-9-(ß-D-arabinofuranosyl)-9H-purin-6-amine [%]	2-Ethoxy-9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-6-amine [%]	additional not determined impurities [%]	All impurities [%] **)
1	8/8	5.57	2.84	0.17	0.20	0.03	<0.01	0.95	9.76
2	32/32	7.32	4.86	0.08	0.08	0.02	<0.01	1.01	13.37
3	8/8	2.35	0.33	0.03	0.09	0.01	<0.01	0.26	3.07
4	16/16	2.76	0.47	0.02	0.08	0.01	<0.01	0.24	3.58
5	24/24	3.02	0.51	0.03	0.10	0.01	<0.01	0.25	3.91

*) injection volume = 2 µl

**) = Values of <0,01 and <0,02 not considered

Test Report

(Experiment 6)

Studies on the stability of fludarabine phosphate under various physical conditions were performed.

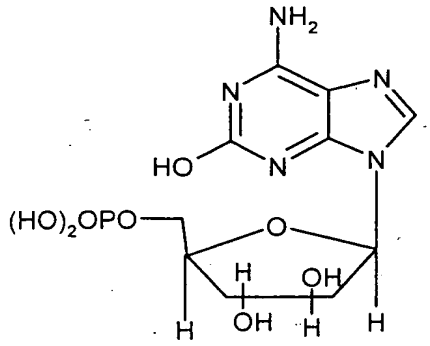
1. General

In the dry state, fludarabine phosphate itself is stable at a high temperature. Fludarabine phosphate decomposes, however, in acid medium and even in alkaline medium. Decomposition also takes place in organic solvents, such as, e. g., ethanol.

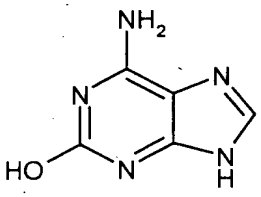
2. Decomposition of Fludarabine Phosphate Based on Temperature and Moisture

At a temperature of 50°C and 60°C and with normal humidity, only a slight decomposition can be seen. If the relative humidity increases, however, a significant decomposition of fludarabine phosphate results.

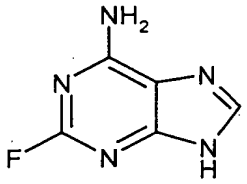
At a temperature of 60°C, fludarabine phosphate decomposes with high humidity or in solution, in particular to the decomposition products C1, C2, and 2-F-Ad.



C1



C 2



2 -F-Ad

6-Amino-9-(5-O-phosphono-ß-D-arabinofuranosyl)-9H-purin-2-ol

6-Amino-9H-purin-2-ol

2-Fluoro-9H-purin-6-amine

Another decomposition product is the previously unidentified substance RRT 0.5.

At still higher temperatures of 70°C, 80°C and 90°C fludara is stable unless exposed to moisture. As soon as higher humidity or even solvent is present, a strong decomposition takes place.

The results of the temperature study are disclosed in the following table:

Temp./Relative Atmospheric Humidity [°C/%]	Time [d]	C1	C2	RRT 0.5	2-F-Ad	Σ Contam-inants
25°C	Start	0.54	0.03	<0.01	0.03	0.91
50	30	0.63	0.07	<0.01	0.06	1.10
50/75	30	0.75	0.27	<0.01	0.04	1.40

Temp./Relative Atmospheric Humidity [°C/%]	Time [d]	C1	C2	RRT 0.5	2-F-Ad	Σ Contam-inants
60	30	0.61	0.10	<0.01	0.07	1.12
60/75	30	1.97	4.70	0.08	0.64	11.78
70	15	0.57	0.10	<0.01	0.09	1.12
70/75	15	9	36	26	<0.1	99
70	30	0.65	0.13	<0.01	0.08	1.19
70/75	30	10	22	42	0.1	99
80	7	0.64	0.12	0.02	0.10	1.23
80	15	0.64	0.33	0.01	0.10	1.47
80	30	0.68	0.16	<0.01	0.09	1.24
80/75	7	9	30	38	0.1	99
90	3	0.59	0.08	0.01	0.10	1.21
90	7	0.64	0.35	0.04	0.11	1.64
90	16	0.64	0.15	0.02	0.09	1.21
90/75	3	6	41	46	0.1	99

RRT 0.5= Peak at relative retention time of 0.5

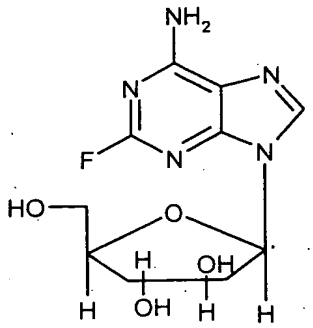
All results in the table relate to % by weight.

Study Parameters:

Column:	150mm HPLC steel column ∅ 4,6mm
Stationary phase:	ChemCosorb 5-ODS- H 5 µm
Mobile phase:	Gradient
	Eluant A: Methanol / KH_2PO_4 (10mmol) 6/94vol.
	Eluant B: Methanol / KH_2PO_4 (10mmol) 20/80vol.
Flow rate:	1ml/min
Wave-length:	UV 260nm
Injection volume:	Assay: 30µl (corresponding to 0.6µg)
	Contaminants/decomposition product
	Solid substance: 4µl (corresponding to 2µg)
	Solutions: 2µl (corresponding to 2µg)

3. Decomposition of Fludarabine Phosphate Based on the pH

A very strong decomposition of fludara takes place in acid solution. The decomposition rate is clearly time-dependent. The main decomposition product is C2 (see above), in addition to C1, 2-F-Ad (see above) and 2-F-ara-A.



2 -F-ara-A

2-Fluoro-9-(ß-D-arabinofuranosyl)-9H-purin-6-amine

In the neutral solution, decomposition can also be observed. The main decomposition product is 2-F-ara-A.

In alkaline solution, a very strong decomposition takes place. In this case, the main decomposition products are C1 and C2, in addition to 2-F-Ad and 2-F-ara-A. Additional decomposition products are the previously unidentified substances RRT 0.4 and at RRT 0.6.

The results are presented in the following table:

Condition	Time [weeks]	C1	C2	RRT* 0.4	RRT* 0.6	2-F-Ad	2-F-ara-A	Σ Contaminants
	Start	0.54	0.03	<0.01	<0.01	0.03	0.01	0.91
Buffer pH 3	1	8.02	6.18	0.06	<0.01	2.99	1.11	18.77
	2	10.27	18.40	0.28	<0.01	5.06	2.19	36.92

Condition	Time [weeks]	C1	C2	RRT* 0.4	RRT* 0.6	2-F-Ad	2-F-ara-A	Σ Con-taminants
	3	11.44	34.37	0.72	<0.01	6.60	3.27	57.27
	4	13.14	53.13	1.42	<0.01	7.74	4.29	80.70
Buffer pH 7	1	0.58	0.04	<0.01	<0.01	0.05	0.92	1.86
	2	0.79	0.05	<0.01	<0.01	0.06	1.89	3.02
	3	0.95	0.06	<0.01	<0.01	0.07	2.93	4.31
	4	1.10	0.07	<0.01	<0.01	0.08	3.78	5.32
Buffer pH 9	1	5.23	1.64	0.03	<0.01	1.81	1.14	10.23
	2	7.30	4.27	0.09	<0.01	3.13	2.33	17.53
	3	8.68	7.24	0.20	<0.01	4.21	3.56	24.42
	4	9.57	10.04	0.34	<0.01	5.09	4.81	30.56
HCl (0.1N)	1	1	43	1	<0.01	17	0.3	61
	2	1	75	4	<0.01	9	0.3	92
	3	0.1	89	6	<0.01	3	0.7	98
	4	0.1	89	9	<0.01	0.8	1	99
NaOH(0.1N)	1	48	3	0.1	30	0.3	0.2	83
	2	51	5	0.1	31	0.3	0.2	90
	3	48	5	0.1	30	0.3	0.2	87
	4	49	7	0.1	31	0.3	0.2	88

RRT 0.4 and RRT 0.6 are peaks at a relative retention time (RRT) at 0.4 and 0.6. All determined values are indicated in % of peak surface area. The amounts of C1, C2, 2-F-Ad and 2-F-ara-A are calculated based on % by weight.

4. Summary

The study results show that fludarabine phosphate is an unusually unstable compound. In particular, very strong decomposition takes place in aqueous solution under acidic or alkaline conditions. Even in the neutral range (at pH 7), there is a clear decomposition.

At elevated temperature and high humidity or in the presence of solvents, fludarabine phosphate decomposes.

Curriculum vitae

Name:	Dr. Thomas Wessa
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Employment History:

July 1999 – current	Chemist at Chemical Development of the Schering AG, Berlin
since September 2001	Chemist on the pilot plant SH, responsible for GMP-compliant drug substance supply of different development and production projects of the Schering AG.
July 1999 – August 2001	Development chemist in process research, responsible for quality control within the automated process optimiziation group.
November 1997 - June 1999	Development chemist in a joint project of the In-Process-Control department of Schering AG, Berlin and the Institute for Instrumental Analytics of the Forschungszentrum Karlsruhe. Subject: "Efficient process control using SAW-sensor analytics"
Jan. 1997 - Oct. 1997	Postdoctorial position at the Institute of Physical Chemistry of the Tübingen University. Member of the working group of Prof. Göpel, responsible for liquid sensing and sensors in HTS.
Juli 1995 - Dez. 1996	Tutor at the Physiotherapy school in Neustadt/Weinstraße; subjects: applied physics and biomechanics.

Education history:

Feb. 1993 - Feb. 1996	Dissertation at Forschungszentrums Karlsruhe at the Institute for Instrumental Analytics. Subject: "Development of a biosensor on the basis of surface acoustic wave devices" under the guidance of Prof. Ache.
July 1992 - Jan. 1993	Diploma thesis at the Institute for Chemical Engineering. Subject: "Model discrimination for the characterization of the diffusion in zeolites" under the guidance Prof. Dr.-Ing. Emig
April 1987 - May 1992	Study of chemistry at the Fridericiana University of Karlsruhe

Berlin, den 31. Januar 2006

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Nationality	**German**

Education and Qualification:

1972-1976	Humboldt-University Berlin Study of Chemistry
1976	Degree
1976-1980	Assistent in the Department Organic Chemistry
1980	Degree Chemistry, Dr. rer. Nat.
1983-1987	Postgradual education : Specialist for medical chemistry / pathological and clinical biochemistry

Employment:

1980-1981	Institute for molecular biology of the academy of science
1981-1989	Institute for pathological and clinical biochemistry, Charite´Hospital, Humboldt-University
Since 1990	Analytical Development, Schering AG

Scientific Activities (Publications, selected papers):

Rabe, H. G. Reichmann, Y. Nakagawa, B. Rüstow and D. Kunze, Separation of Alkylacyl-and Diacyl Glycerophospholipids and their Molecular Species as Naphthylurethanes by HPLC J. Chrom. 493 (1989) 353-360

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